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SURFACE SAMPLING-BASED DECONTAMINATION STUDIES AND PROTOCOL FOR DETERMINING SPORICIDAL EFFICACY OF GASEOUS FUMIGANTS ON MILITARY-RELEVANT SURFACES

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A major consequence of biological terrorism in a military setting is the wide degree of contamination of combat/protective equipment used by war-fighters and first responders. Appropriate selection of a decon technology, successful implementation, and re-use of assets following decontamination rely principally on extensive pre- and post-decontamination sampling. One of the challenges related to the Test & Evaluation demonstration of decon products has been poor recovery of biological contaminants from complex surfaces. Here, we report the optimization of surface sampling for quantifying biological contaminants. An optimized surface sampling protocol was devised and used in decontamination studies for determining sporicidal efficacy of two fumigants on contaminated military-relevant surfaces.

15. SUBJECT TERMS

Surface sampling	Spores	Fumigants	Decontamination
B. anthracis	*B. subtilis*	Antimicrobials	Vaporous Hydrogen Peroxide
Chlorine Dioxide Gas	Sporicidal		

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PREFACE

The work described in this report was authorized under Project No. BA06DEC414. The work was started in October 2006 and completed in March 2008.

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Use of the chamber and CD generator acquired from U.S. Environmental Protection Agency's National Homeland Security Research Center funds is greatly acknowledged here.

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CONTENTS

FIGURES

TABLES

SURFACE SAMPLING-BASED DECONTAMINATION STUDIES AND PROTOCOL FOR DETERMINING SPORICIDAL EFFICACY OF GASEOUS FUMIGANTS ON MILITARY-RELEVANT SURFACES

1. INTRODUCTION

Bio-warfare agents (BWA) encompass a broad-range of replicating organisms such as bacterial spore formers (e.g., *Bacillus anthracis*), non-spore formers (e.g., *Francisella tularensis* and *Yersinia pestis*), and non-replicating toxins (e.g., ricin and botulinum). Spores of *B. anthracis*, the causative agent for anthrax in humans and animals are known to withstand extreme environmental insults, such as heat, UV light, desiccation, and, therefore, are regarded as one of the most resistant BWAs. The infective dosage has been reported to be a few to several thousands spores, depending on the age, sex, and immune system (Inglesby *et al.*, 1999). Dissemination of *B. anthracis* spores via the U.S. Postal Service in 2001 not only resulted in the deaths of several civilians, but also in the contamination of a number of commercial and government office buildings (Canter, 2005). The clean up of several hundred thousand cubic-feet of three-dimensional space within such buildings required a concerted effort involving the private sector, governmental agencies, and academia. The U.S. Environmental Protection Agency (EPA) was responsible for the remediation and restoration of these contaminated buildings. The critical issues facing the daunting clean up efforts follow:

a) Quantitative methodologies for assessing the surface contamination levels on building interiors and equipment surfaces.
b) Sporicidal technologies effective in reducing the surface contamination (to zero) of three-dimensional space within large buildings.
c) Quantitative methodologies for confirming the effectiveness of decon technologies by sampling environmental surfaces.
d) A consensus on desired decontamination standards for re-occupancy of the buildings after a clean up operation.

Reliable methodology for assessing the pre- and post-decon contamination levels and effectiveness of a cleanup technology is absolutely vital to post-event restoration efforts. Biological sampling from environmental surfaces has typically been performed using swabs, wipes, or vacuum filtration/collection (CDC, 2002; Sanderson *et al.*, 2002, Rose *et al.*, 2004, and Hodges *et al.*, 2006). Well-developed and validated sampling methods are currently lacking, especially from complex porous surfaces (GAO, 2005 and Brown *et al.*, 2007). Furthermore, no sampling-based decontamination protocols are currently available to assess the effectiveness of a given technology on either building-relevant or military-relevant materials.

The antimicrobial products used for environmental surface clean up are registered and regulated by the EPA Office of Pesticide Programs. Registration of these products relies on efficacy data generated using one or more of the standardized sporicidal test methods, such as AOAC Sporicidal Activity Test (SAT), AOAC® *Official Method*™ 966.04, ASTM 2414-05[1], or ASTM 2197-02[2], demonstrating >6-log spore kill of *B. anthracis,* or an appropriate surrogate (e.g., *B. subtilis*). All of these methods are based on testing hard surface carrier-based spores, which are submerged in disinfectant for a desired contact time, followed by the addition of a neutralizer and enumeration of viable spores recovered from the carrier. Almost all standard test methods use small-size coupons, e.g., 5x5 mm squares or 1 cm disc, on which ~1-10 million spores are inoculated. While the AOAC-SAT is qualitative, the latter two test methods are quantitative, and provide the log reduction estimates.

The quantitative test methods listed above are appropriate and best suited for initial screening of the antimicrobial or bio-decontamination products and for regulatory purposes. However, such tests do not provide information on whether similar efficacy of decon products will be observed when using coupons of larger size (2-10 cm in size) or tests with large panel size (12 $in.^2$), or even complex large size equipments. Within the DoD community, as a given technology matures from TRL4-5 to TRL6-7, efficacy of a given decon product must be demonstrated on large-size panels and/or 'real-life' equipment in operational environment. Acquisitions programs rely on systematic evaluation of the efficacy of decon products from screening level testing to large panels/full-size equipment. The acceptable levels of decontamination may differ from civilian setting to bioterrorism related scenarios related to US armed forces around the world. In the civilian setting, complete decontamination is the only accepted criteria before a building is turned over for re-occupancy (Canter, 2005). Surface sampling of biological materials before and after a cleanup operation is at the very core of such BW sampling assessment. Until 2005, a lack of validated sampling methods posed a serious challenge to development of efficient sampling methodologies for BW agents (see GAO Report 2005 on Anthrax Detection).

After initial laboratory screening and testing of sporicidal chemicals, their effectiveness must be demonstrated in field testing of equipment and complex surfaces. Efficacy can only be shown if adequate sampling protocols are available. No decon test protocols based on surface sampling were available at the start of this program. The long-range goal of this program was to develop a sampling-based bio-decon protocol for assessing the efficacy of liquid disinfectants and gaseous fumigants. This protocol is expected to feed into the development of an updated test operating procedure (TOP) for the Test & Evaluation community within the DoD. Such a TOP will not just involve protocols based on suspension tests, but also a sampling-based decon approach proposed to be developed under this program. A separate report on the efficacy evaluation of liquid disinfectant is being prepared and submitted by our collaborators led by Lindsay Sobota in NSWC, Dahlgren. Here, we summarize our results on a) optimization and development of a sampling-based decon protocol and b) sporicidal efficacy evaluation of two common fumigant technologies, chlorine dioxide (CD) gas and vaporous hydrogen peroxide (VHP) against two inoculants, *B. subtilis* and *B. anthracis* ΔSterne, on four military-relevant surfaces using such protocol.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

The bacterial strains used for these studies were plasmid-free avirulent *Bacillus anthracis* (ΔSterne; DOD Unified Culture Collection, UCC # BAC1056) and *B. subtilis* (American Tissue Culture Collection, ATCC #19659).

2.2 Media and Culture

Both test organisms were grown in Tryptic Soy Broth (TSB) at 37 °C. Desiccated cultures received from UCC and ATCC were reconstituted in TSB for culturing. Broth cultures for each test organism were streaked for single colonies on Tryptic Soy Agar (TSA) plates and single colonies were inoculated into TSB and grown overnight at appropriate temperature. Gram stain and spore staining were performed to confirm broad microbiological characterization. The overnight broth cultures were supplemented with sterile glycerol to a final concentration of 20% (v/v) and maintained at -80 °C for long-term storage.

2.3 Spore Preparation

Spores were prepared from both test inoculants (*the spore preparation protocol is the same for all organisms).* A glycerol stock was streaked for a single colony on a TSA plate. Following an overnight incubation at 37 °C, a single colony was inoculated into 5 mL TSB media. This culture was grown for 16-18 hr at 37 °C, and a 250-µL aliquot of this overnight broth culture was spread onto 20 large Lemko agar plates. These plates were wrapped in parafilm and incubated at 37 °C. A wet mount slide was prepared using bacterial culture from each plate every 24 hr to assess sporulation progression. The plates were harvested when percent sporulation reached ≥85%.

The spore population on the plates was recovered by adding 25 mL sterile distilled water to one of a set of four plates. The spores were gently dislodged off the plate using a cell spreader. The spore suspension collected from a set of four plates was then transferred to a sterile centrifuge bottle. After recovery and pooling from all 20 plates, two additional washes with 25 mL sterile water were used to wash off the residual spores from the plates and added to the pooled suspension. The spore suspension was centrifuged at 6000 rpm for 30 min. The supernatant was discarded, and the pellet was resuspended in 150 mL sterile water. This wash step was repeated two times, with resuspension of the pellet each time. After the last wash, the pellet was resuspended in 100 mL 70% ethanol, and incubated at ambient temperature on a rotator for one hour. This step eliminated carry-over of vegetative cells (if any) in the spore prep. The suspension was then re-centrifuged, and the pellet was resuspended in 100 mL of sterile distilled water. The suspension was incubated at 65 °C for 30 min to rid the prep of proto-spores and any residual vegetative cells. The heat-treated spore suspension was centrifuged, the supernatant was discarded, and the pellet was resuspended in 50 mL sterile distilled water. A spore stain was performed to microscopically confirm the quality of the prep. Presence of >85% spore in microscopic analysis was an acceptable criterion for the spore quality.

The spore preparation was enumerated by making ten fold serial dilutions in BPW and plating an aliquot of 0.1 mL from 10^{-6} and 10^{-8} dilutions in triplicate on TSA plates. The colony forming units (CFU) were counted following an overnight incubation at 37 °C. The spore stock was diluted to a titer of ~$4x10^9$/mL titer and 1 mL was aliquoted in sterile eppendorf tubes. For long-term storage and ensuring minimal losses in viability, the spores were pelleted, 0.6-mL of supernate was discarded, and the tubes were then stored in a -20 °C freezer.

As required, 2 or 4 frozen tubes (depending on final volume required) were thawed on ice and 0.6 mL sterile distilled water was added to each tube. The contents were then pooled in a sterile Falcon tube and a measured volume of sterile distilled water was added to prepare a working stock with a titer of $4x10^8$/mL. Equal volume of spore suspension and 1% sterile fetal bovine serum solution (FBS) was mixed to generate a working stock with $2x10^8$/mL titer containing 0.5% serum protein. The latter was added to represent organic burden or bio-burden.

2.4 Coupon and Panel Materials

Bulk quantities of the four military relevant materials, butyl rubber, polycarbonate, glass, and chemical agent resistant coating (CARC) painted steel were procured from high volume retail sources (Table 1). Small-size pieces (henceforth referred to as coupons) used for extraction optimization and sampling screening were cut to 2 x 5 cm size, by the Advanced Design and Manufacturing Team, Engineering Directorate, at ECBC, APG, MD. Large panels (30.5 x 30.5 cm) were used for sampling. Table 1 summarizes the material source information.

Table 1. List of Vendors for Test Materials, Wipes, and Swabs Used in Study

Material	Vendor	Catalog
Polycarbonate	E.J. Enterprises	0.177"thick Sheet 4'x8'
Steel	Durrett Sheppard	11 Gauge A572 Grade 50 sheet 4'x8'
CARC paint	Automated Coatings	Primed and painted per MIL-C-53039A 383 Green
Butyl rubber 1/8" thick, 12"x12"	McMaster Carr	8609K35
Fire rated glass 12"x12"	McMaster Carr	8481K74
Sterile cotton swabs	VWR/Puritan	10805-154
LP polyester wipes	VWR	TWTX3211
Sterile Foam tipped Applicators	VWR	10806-036
Cotton wipes	VWR	21902-985

2.5 Coupon/Panel Handling and Sterilization

All four material types, both small coupons and large panels, were washed with soap and water, rinsed with water, sprayed with 70% ethanol and then allowed to dry at room temperature before use. The small coupons were placed in Petri dishes and then autoclaved for 30 min with a 10 min dry cycle. The large panels were individually autoclaved in sealed sterilization pouches (VWR, Inc.) by autoclaving for 90 min with a 10 min dry cycle.

2.6 Spore Inoculation

Approximately 16-20 hr before a test run, coupons were inoculated with an aliquot of 50 µL as either five distinct 10 µL spots, one single drop, or one single drop spread across the surface. In addition, for sampling, an aliquot of 1 mL spread (10^7 total spore number on small coupons), or 10 mL spread (6.25×10^8 total spore number on large panels) across the surface. The spore working stock of both inoculants contained 0.5% FBS as bio-burden. The coupons were dried overnight in a bio safety level 2 hood. Titer enumerations were performed with each test run, with 50 µL containing ~10^7 spores added directly into 20 or 25 mL extraction media.

2.7 Spore Extraction

Spores were extracted from small coupons by physical treatments. In the initial phase of the study, a number of parameters - extraction media, vortexing, sonication, combination of vortexing + sonication, and inoculation method - were systematically investigated for optimal spore recovery off the coupon surface. The extraction media tested were water, phosphate buffer saline (PBS), TSB ($1/10^{th}$ strength), and buffered peptone water (BPW) $1/4^{th}$ strength, all with and without 0.05% Tween 80 added. Inoculated coupons were transferred to 20 mL recovery media in a 50 mL sterile Falcon tube. The

samples were sonicated for 10 min in a Branson™ sonicator and then vortexed for 2 min using a high-capacity pulse vortexer (Q Glass Co., NJ) at ~1500 rpm.

Optimization of physical parameters included comparison of sonication times (5, 10, or 15 min) and vortexing times (1, 2, or 5 min). Furthermore, effect of vortexing alone (2 and 5 min), sonication alone (15 min), or 15-min sonication + 2-min vortexing were also investigated.

2.8 Surface Sampling

Sampling optimization was performed using small coupons. Two types of wipes, polyester and cotton, and 2 types of swabs, cotton and foam, were compared. At first, spore recovery by sampling was determined using wipes and swabs individually, and then sampling was performed with a combination of one wipe and three successive swabs. On all four large panel types, twenty sectors (2 x 5 cm in size) were inoculated. Four sectors were sampled before fumigation (as positive controls) and 16 sectors were sampled after fumigation (as test samples). For spore recovery and fumigation studies, each sector on large panel was sampled by a combination of one moistened polyester wipe, which was put into 20 mL extraction media, followed by three moistened cotton swabs, which were all put into a separate tube containing 10 mL extraction media. The tubes containing wipes or swabs were vortexed for 2 min, followed by enumeration of spore titer in each sample.

2.9 Dilution Plating and Enumeration

Typically, for control sets, ten-fold dilutions were made between 10^{-1} and 10^{-4} (wipes) and between 10^{-1} and 10^{-3} for swabs. An aliquot of 100 µL was spread plated on triplicate TSA plates. For fumigated samples, an aliquot of 100 µL was spread plated on triplicate TSA plates and a 3-mL aliquot was pour plated on two plates, using TSA media equilibrated to 55 °C. The poured plates were allowed to solidify at room temperature for 2-3 hr. All spread and pour plates were then incubated at 37 °C overnight. The colony forming units (CFUs) were counted using a Qcount™ plate counter (Spiral Biotech; Norwood, MA). The CFU from the three replicate plates were averaged and multiplied with dilution factor, volume factor, and total volume of the extraction media to calculate CFU/coupon.

2.10 Fumigant Generators and Fumigation Chamber

The test chamber used in these fumigation studies was procured from ClorDiSys, Inc. It was 8 cu. ft in dimension (2 ft x 2 ft x 2ft), and made of 316-grade stainless steel (Figure 1). The chamber had one window for observation, a door for sample introduction, and four ports on the front panel for sensor placement. All glass surfaces were covered when used with CD gas, since CD is light sensitive. This system can be safely utilized to test a wide variety of decontamination gases and vapors. The same chamber was utilized for testing with vaporous hydrogen peroxide (VHP) and chlorine dioxide (CD) gas, and was equipped with temperature, relative humidity (RH), pressure, and fumigant sensors. Additionally, the test chamber was equipped with five ante-chambers for easy access and removal of Petri plates containing inoculated coupons. Each ante-chamber had an inner and outer airlock door. After fumigation for pre-specified time, a Petri plate was placed in one of the ante-chambers by opening and closing of the inner door. The Petri plate was removed by opening the outer door without affecting the process parameters for remaining coupons in the test chamber. The chamber was filled with CD at a flow rate of 20-L/min and the concentration of CD was maintained near constant by a PLC-controlled sensor regulator installed in the generator. The VHP concentration was largely dependant on the RH (must be <35% in the test chamber) and the M-100 (Steris, Inc.) generator was used to pump 1.1 g/min VHP at a rate of 5 cfm. The VHP concentration was measured using a Drager Polytron sensor.

Three circulation fans installed in the chamber mimicked the air circulation effected by fans in commercial large-room decontamination. Gas circulation in the chamber ensured even distribution of fumigant over the exposed surfaces. The chamber temperature and RH was programmed, controlled, and monitored. The CD gas sensor was a custom-built UV-VIS spectrophotometer and the signal was measured at two frequencies (proprietary), one quantifying the CD signal and the other quantifying the background noise. The sensor's sensitivity was between 1-30 mg/L $\pm$ 5% range. The CD concentration was also confirmed by chemical method, i.e., the iodometric titration method.

The CD gas from ClorDiSys technology was generated by passing chlorine gas over a series of three cartridges containing sodium hypochlorite salt. The Cloridox-GMP generator was equipped with real-time monitoring of common process parameters, i.e., pressure, temperature, and gas concentration. The gas concentration was controlled by regulating the flow rate, feed concentration, real-time measurement using a spectrophotometer, and PLC valve. Typically, a cycle with desired CD concentration was programmed with pre-conditioning (RH ramps to 75%), conditioning (30 min at 75% RH), charging (CD conc. ramps to the set point), exposure, and aeration phases.

The VHP was generated using a Steris VHP M100 generating system using 35% HP. The control of parameters such as airflow and peroxide solution feed rate was through a Siemens OP-7 interfacing unit. The desired VHP concentration at the selected flow rate was fed into the interfacing unit, which calculates and sets the peroxide solution feed rate to achieve the desired VHP concentration. Decontamination technology of VHP requires relatively low humidity conditions to minimize the likelihood of condensation. The low humidity was maintained by drying the air with a Munters MG90 desiccant dehumidifier before it was fed into the VHP delivery system.

2.11 Fumigant Exposures

The VHP concentration x time (CT) ranged between 948 and 1320 ppmv.hr. The coupons were exposed to 375-450 ppmv VHP for 3 hr. The CD concentration was on average 3500 ppm/hr and ran for about 2.6 hr. The CT values for CD gas on average were 9000 ppmv.hr. Six fumigation runs were performed with each fumigant and each inoculant (*B. anthracis* ΔSterne and *B. subtilis*).

2.12 Efficacy Determination and Recovery Efficiency

Log values for the four replicate coupons (small coupons used for extraction and sampling optimization) were computed and averaged to get the mean log (CFU). Standard deviation was also computed using the same replicate values. For recovery experiments, each experiment was repeated twice by a set of two analyst pairs. For efficacy experiments, a set of six experimental repeats were performed by a set of four analysts using the sampling protocol, with each analyst sampling one of the four panel types. Spore recovery efficiency was calculated by taking the percentage of the ratio of the spore number recovered/the spore number inoculated on each coupon (titer enumeration). Log reduction values were calculated by subtracting the mean log CFU of the fumigated samples from that of the control samples.

3. RESULTS

3.1 Spore Quality Used for Working Spore Stock Preparation

Production and use of high-quality spores are critical to the development of quantitative decontamination methods for assessing efficacy of sporicidal agents. Spores of both inoculant types, *B. subtilis* and *B. anthracis* (ΔSterne), were prepared as detailed in Materials and Methods. A quality assessment was performed on both spore preparations used in this project. Spore quality was assessed as a qualitative ratio of spores:vegetative cells, by microscopic analysis of spores stained with malachite green spore stain. As shown in Figures 1 and 2, both preparations possessed greater than 95% spores. A working stock of each spore inoculant was prepared by mixing equal volumes of spore suspension with a titer of $4x10^9$/mL and 1% fetal bovine serum, resulting in a working spore stock with a titer of $2x10^8$/mL containing 0.5% serum as organic burden. The titer of each spore working stock was enumerated before use and found to be ~2x108/mL (results not shown). Since an aliquot of 50 µL was used for inoculation, typical spore challenge level was $10^7/10$ cm^2.

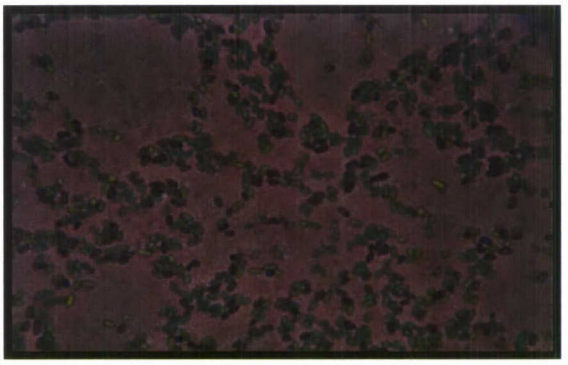

Figure 1. Stained Spore Image of *B. anthracis* ΔSterne Spores

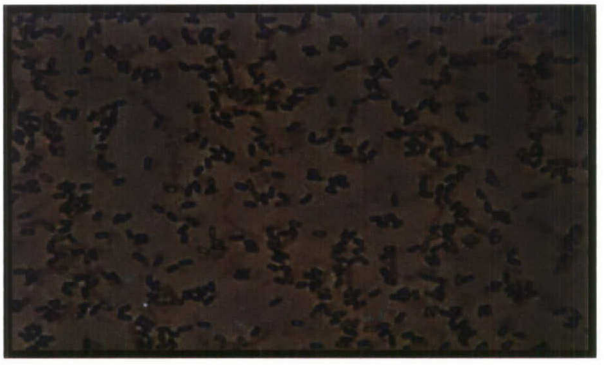

Figure 2. Stained Spore Image of *B. subtilis* Spores

3.2 Analyst Variability Assessment

The scope of this program was very broad, with use of four replicates per data point per material type (four in all) with at least two experimental repeats (optimization section), four experimental repeats (spore recovery by sampling), or six experimental repeats (fumigant efficacy assessment) resulting in the use of a large number of small coupons or large panels. This necessitated involvement of at least four analysts per experiment. Assessment of inter-analyst variability is crucial to ensure confidence in the results and ensure lack of bias in the data generated by multiple analysts. Prior to the

commencement of this program, an inter-analyst variability assessment was performed to determine the degree of variability among all laboratory analysts participating in this project. Each analyst diluted and plated enumerations for five control samples, where approximately 10^7 spores were added directly to the extraction buffer. Each analyst also plated from a set of pre-diluted tubes prepared by one analyst (VR). In addition, each analyst performed extractions from five replicate coupons inoculated with 10^7 spores dried on the surface. The results from the inter-analysts variability assessment, summarized in Figure 3, show extremely low variability among the seven analysts for each of the three sample types. The average log spores recovered from the control samples, extracted samples, and VR pre-diluted tubes ranged between 6.86-7.02, 6.60-7.02, and 6.69-7.11. These results established an acceptable and comparable level of competency in quantitative assessment of microbiological samples by different analysts.

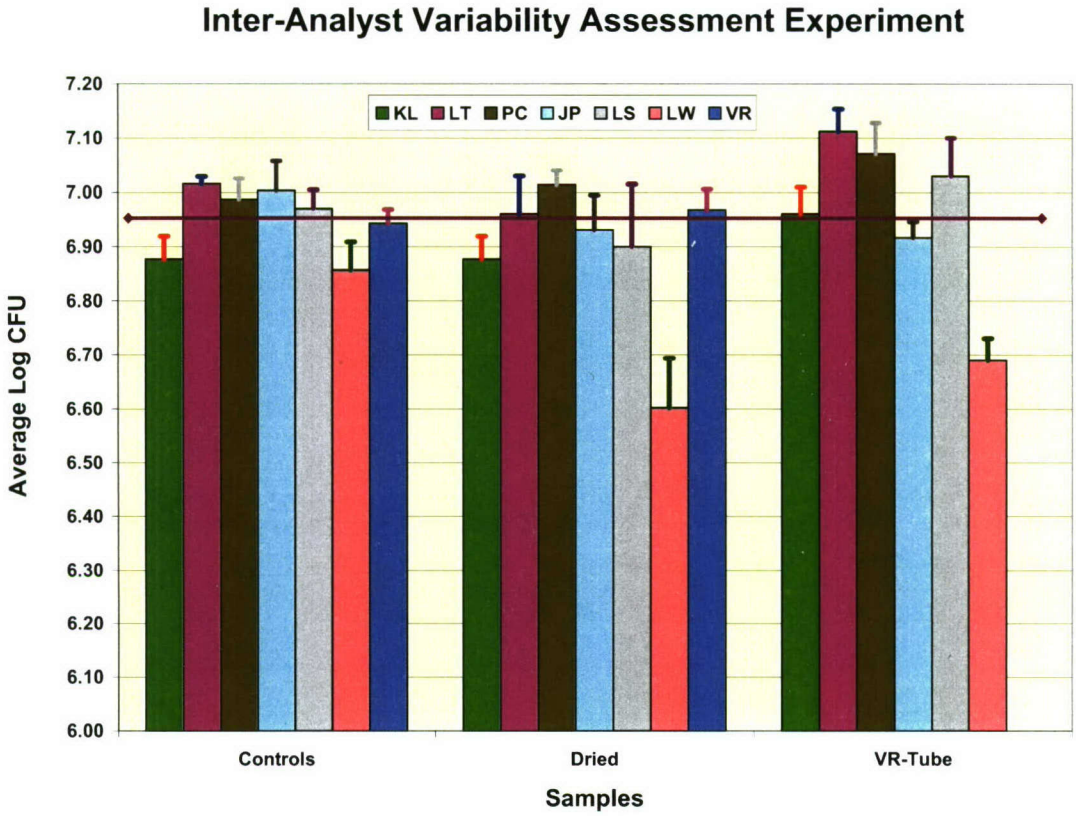

Figure 3. Inter-Analyst Variability Assessment as Expressed by Mean log (CFU). The horizontal bar across the plot shows the mean log CFU for all data points. Note the expanded scale on Y-axis to better depict the subtle differences in log CFU estimation by different analysts

3.3 Optimization of Spore Extraction from Small (2 x 5 cm) Coupons

Spore inoculation and efficient extraction of spores from porous and non-porous surfaces is vital to decon protocol development. Spore density (spore number per unit area) can result in layering and clustering over a surface area, which can impact spore recovery. In addition, spore recovery can be affected by three parameters: a) the innate nature of the surface, e.g., porosity and surface charge; b) the type and volume of extractant used; and c) physical agitation, i.e., sonication and vortexing to dislodge the spores off the surface. A single tube method (STM) developed under the auspices of EPA-funded 'Systematic Decon' program was adapted for this program. In this section spore extraction parameters listed were evaluated for selecting the optimal conditions for spore extraction from small coupons.

3.3.1 Effect of Extraction Media and Use of Surfactant

Four common extraction media, dilute TSB, BPW, PBS and water, were screened. Each extractant was used in the absence and presence of 0.05% Tween-80. As shown in Figure 4, spore recovery was comparable for all four extraction media lacking the surfactant. Furthermore, the student's t-test analysis (p value of 0.01) showed no significant difference among the four. However, the results show consistently higher recovery of ΔSterne spores from glass, CARC-painted metal, polycarbonate, and rubber coupons with extraction media containing Tween-80. In the absence of surfactant, relatively lower recoveries were observed from all four coupon types, especially from CARC-painted steel and glass. Statistical t-test analysis (at a p value of <0.010) confirmed that the spore recovery in the presence of surfactant was significantly higher than in the absence of surfactant. In general, spore recoveries ranged between 20-80%. The data also showed standard deviation of <0.2 log CFU for eight replicate data points (two experimental runs with four replicate coupons in each run), indicating low variability and high reproducibility for spore recovery. Based on these results, BPW containing tween-80 was selected as the preferred extraction media for further study.

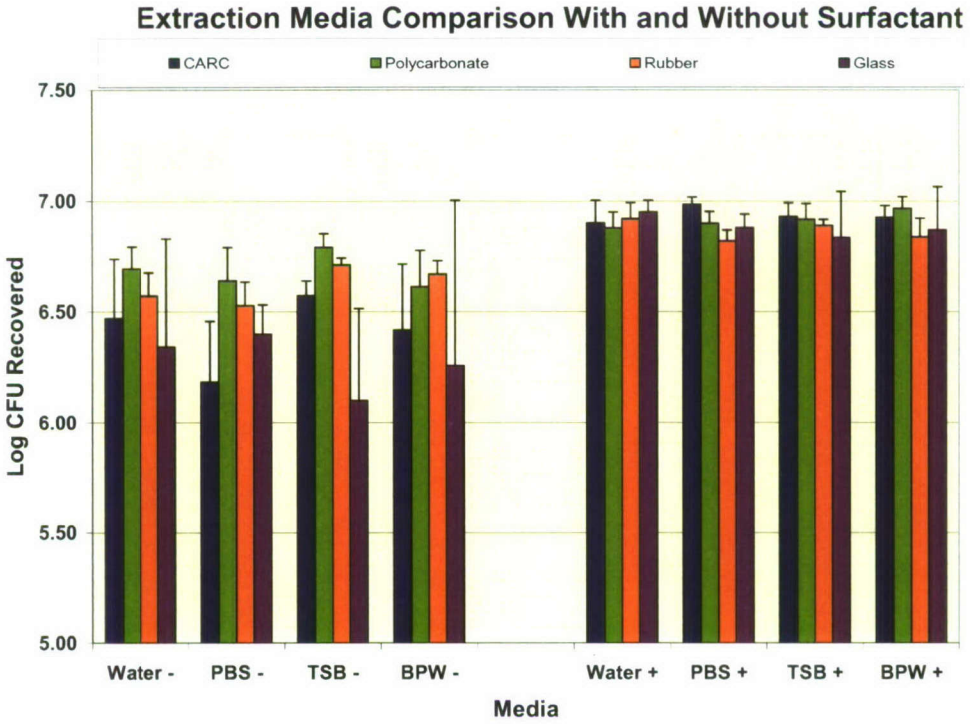

Figure 4. Effect of Four Extraction Media (+/- tween-80) on Spore Extraction from Four Surface Types. Each colored bar represents a different material, with (-) and (+) representing without and with surfactant, respectively. Please note the expanded scale on Y-axis to better depict the subtle differences in log CFU recovered in different media

3.3.2 Inoculation Methods

Three inoculation methods were evaluated to determine if this parameter affected spore number recovered by extraction. The spore inoculations were done as follows: ~7 log spores in one 50-μL drop, five 10-μL droplets, or one 50-μL drop spread across. The three different approaches resulted in three spore densities. The results (Figure 5) show that spore recovery is only marginally affected by the three inoculation methods. Student's t-test analysis (p value of 0.01) of the data confirmed

that the spore recoveries were not statistically different. Based on these results, five 10-μL droplets were selected as the preferred inoculation method for further study.

3.3.3 Vortexing Times

The effect of three vortexing times (1, 2, and 5 min) on spore recovery was investigated for each material type (Figure 6). Overall, the results showed comparable spore recovery for each of the three vortexing times with only marginal differences. A t-test analysis confirmed such differences were not statistically significant. A two minute vortexing time was selected for future extraction/sampling experiments.

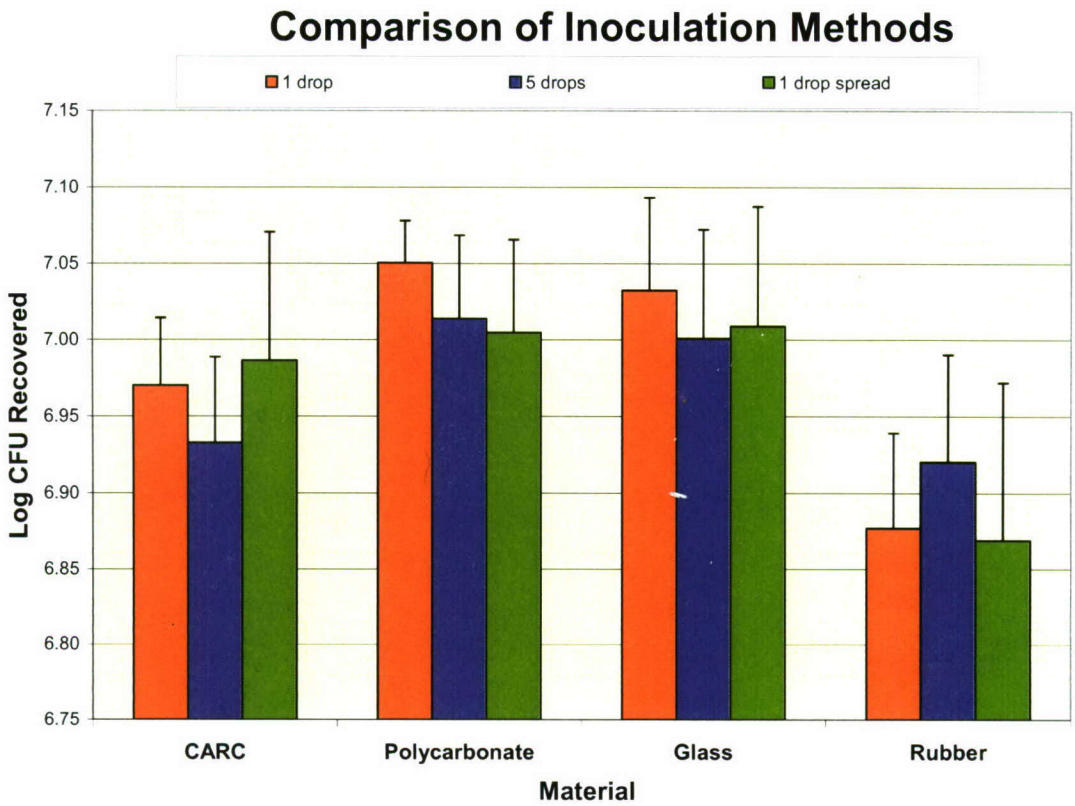

Figure 5. Effect of Three Inoculation Methods on Spore Extraction. Average log CFU recovered for each inoculation method is shown for the four material types. Please note the expanded scale on Y-axis to better depict the subtle differences among log CFU recovered

3.3.4 Sonication Times

The effect of sonication time on extraction of spores from the four material types was analyzed. Three sonication times (5, 10, and 15 min) were compared in terms of average spore recoveries. Similar to the vortexing times, the three sonication times resulted in comparable spore recovery, with no statistically significant differences (Figure 7). Based on these results, 10-min sonication was selected for spore extraction from small coupons.

3.3.5 Spore Recovery Efficiency of *B. subtilis* versus *B. anthracis* ΔSterne

Similar to the optimization experiments performed for *B. anthracis* ΔSterne at ECBC, collaborators at the Dahlgren Naval Surface Warfare Center (NSWC) devised an optimized protocol for *B. subtilis* spores, in which water was chosen as the preferred extractant. Experiments were performed at ECBC to corroborate the NSWC optimized protocol. Inoculated spores of *B. subtilis* on small coupons were extracted with water or BPW containing Tween 80. The extraction was achieved by 2-min vortexing (determined by NSWC to be optimal for spores lacking serum) or the combination of 2-min vortexing + 10-min sonication (determined by ECBC to be optimal for the extraction of ΔSterne spores containing 0.5% serum). The results showed that *B. subtilis* spores containing 0.5% serum were efficiently extracted with water just by vortexing. However, it was found that BPW with Tween 80 resulted in improved recovery of *B. subtilis* spores from four surface types (data not shown).

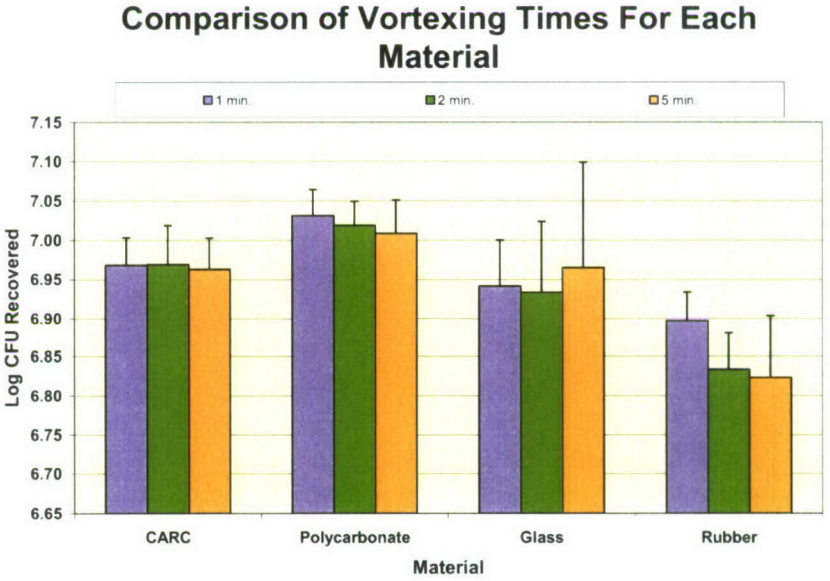

Figure 6. Effect of Different Vortexing Times on Spore Extraction. Average log CFU recovered for each vortexing time is shown for the four material types. Please note the expanded scale on Y-axis to better depict the subtle differences log CFU recovered

3.4 Optimization of Spore Recovery by Sampling from Small (2x5 cm size) Coupons

The major emphasis of this program was to develop a quantitative sampling-based decon method for assessing efficacy of gaseous fumigants (conducted by ECBC, Edgewood group) and liquid disinfectants (conducted by NSWC, Dahlgren group). As mentioned in section 1, validated sampling methods were not available at the commencement of this program. As a first step towards the long-term goal, wipes and swabs were screened for their ability to remove spores off the four surface types. In addition, different inoculation approaches and physical treatments, such as vortexing and sonication (or combination thereof), were compared for their effectiveness in loosening of the spores from sampling materials, swabs, or wipes. In this section, results from experiments aimed in sampling optimization are summarized below

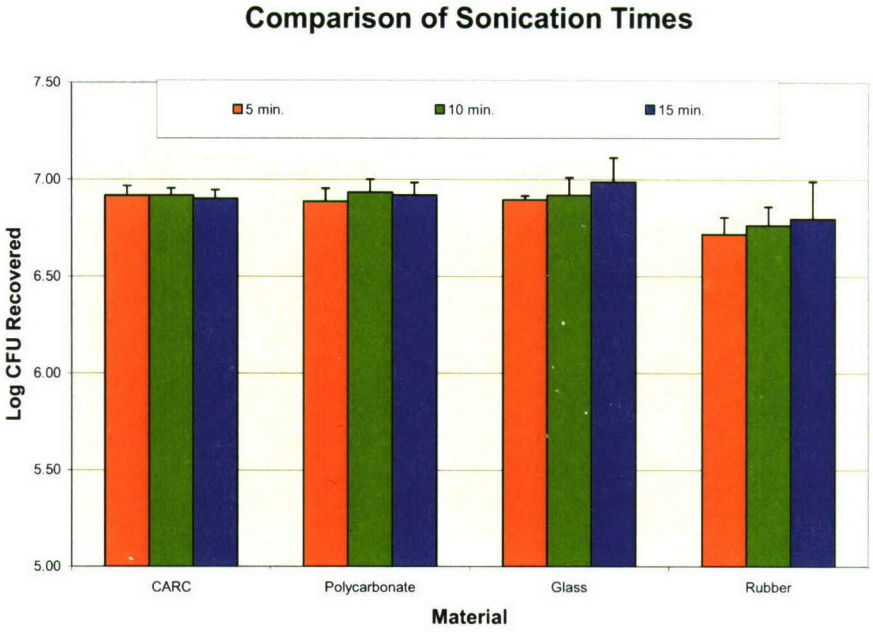

Figure 7. Effect of Sonication Times on Spore Extraction. Average log CFU recovered for each sonication time is shown for the four material types. Please note the expanded scale on Y-axis to better depict the subtle differences in log CFU recovered

3.4.1 Screening of Swabs and Wipes and Residual Spores on Coupons

Two swab types (cotton and Macrofoam) and two wipe types (cotton and polyester) were compared with respect to their ability to remove the spores from different coupon types. Cotton swabs and polyester wipes resulted in consistently high spore recoveries, particularly from polycarbonate. The spore recovery was consistently low with cotton wipes, with a range between 28-48% (Table 2). The t-test analysis showed that the spore recoveries observed with polyester wipes, cotton swabs, and foam swabs were all statistically significant compared to that with the cotton wipes. Statistical analysis also showed that compared to foam swabs, recoveries with polyester wipes was significantly better from polycarbonate and glass surfaces.

For this set of experiments, residual spores left on coupons were assessed by extracting the spores from the coupons, which had already been sampled. This was done to provide insight with respect to the number of spores that are not recovered from the swab or wipe, specifically whether these spores remain primarily on the coupon or are simply adhered to the sampling material. The results from these extracted coupon samples, shown in Figure 8, showed that a significant number of spores remain on the sampled coupons, which suggests that multiple wipes or swabs might result in higher recovery.

3.4.2 Optimization of Spore Release from Wipes and Swabs

Based on the results from the sampling material comparison, cotton swabs and polyester wipes were selected for further analysis. Experiments were performed with a porous surface, CARC-painted steel, and a hard non-porous surface, glass, to see the effect of two common physical treatments on spore release from wipes and swabs. Three conditions were compared: two minute vortexing, ten minute sonication, and two minute vortexing + ten minute sonication. The results, as summarized in Table 3, show that spores are effectively released by all three treatments and no statistically significant difference in spore recovery was observed for the three conditions investigated.

Table 2. Comparative Spore Recovery with Swabs and Wipes

Material	Cotton Wipe	Polyester Wipe	Cotton Swab	Foam Swab
CARC	29	34	33	33
Polycarbonate	48	71	65	58
Rubber	28	48	56	50
Glass	47	62	42	48
Range	***28-48***	***34-71***	***33-65***	***33-58***

3.5 Spore Sampling from Large Panels

An initial large panel recovery experiment was performed with two cotton swabs or polyester wipes using the optimized protocol. Five pre-marked locations (2 x 5 in.) were inoculated with 10^7 spores in a 50-µL aliquot. Relative to small coupons, the results showed comparable spore recovery from large panels, with low standard deviations (Figure 9). As expected, the lowest spore recovery was observed for CARC-painted steel.

3.5.1 Effect of Large Area Inoculation on Sampling from Large Panels

The issue of how large area inoculation on large panels could affect spore recovery was investigated in next set of experiments. As described in Materials and Methods, a 10 sq in. (or 625 sq cm) area was inoculated with a spore suspension containing 70% ethanol (approximately 6x10E8 spores total) with a 10 mL aliquot spore suspension spread over 10 sq in. area. Five pre-marked sampling areas (2 x 5 cm) were sampled with cotton swabs or polyester wipes. The recoveries were computed and compared to the predicted number of spores from five sampled areas to assess deviation and variability in the sampled areas and among the four panel types: glass, polycarbonate, rubber, and CARC-painted steel. The results on the basis of comparative log CFU and percent recoveries using cotton swabs are summarized in Figures 10 and 11. The results show low recoveries from CARC-painted steel, and high recoveries from glass. Furthermore, spore recovery showed a 10-fold variability, ranging between 5x10E5 and 5x10E6 per sampled area. Based on this result, large area inoculation (even though more realistic) was not pursued any further because of high variability. Inoculation with five 10-µL droplets containing 10^7 spores was determined to be the preferred inoculation method for further experiments.

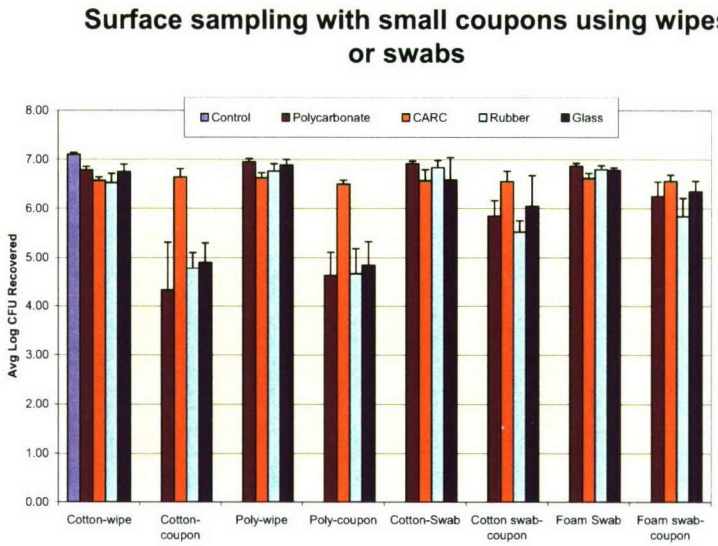

Figure 8. Relative Distribution of Spores on Coupons and Sampling Materials from Different Surfaces. Bars represents spores recovered with the sampling material (cotton wipe, polyester wipe, cotton swab, and foam swab) and the residual spores on the coupons following sampling

Table 3. Release of Spores from Wipes and Swabs by Sonication and/or Vortexing

Mean LOG CFU Recovered			
2 Cotton Swabs			
	2-min vortex	**10-min sonication**	**Both**
CARC	6.80	6.76	6.84
GLASS	6.85	6.79	6.85
Polyester Wipe			
	2-min vortex	**10-min sonication**	**Both**
CARC	6.82	6.82	6.83
GLASS	6.98	6.97	6.95

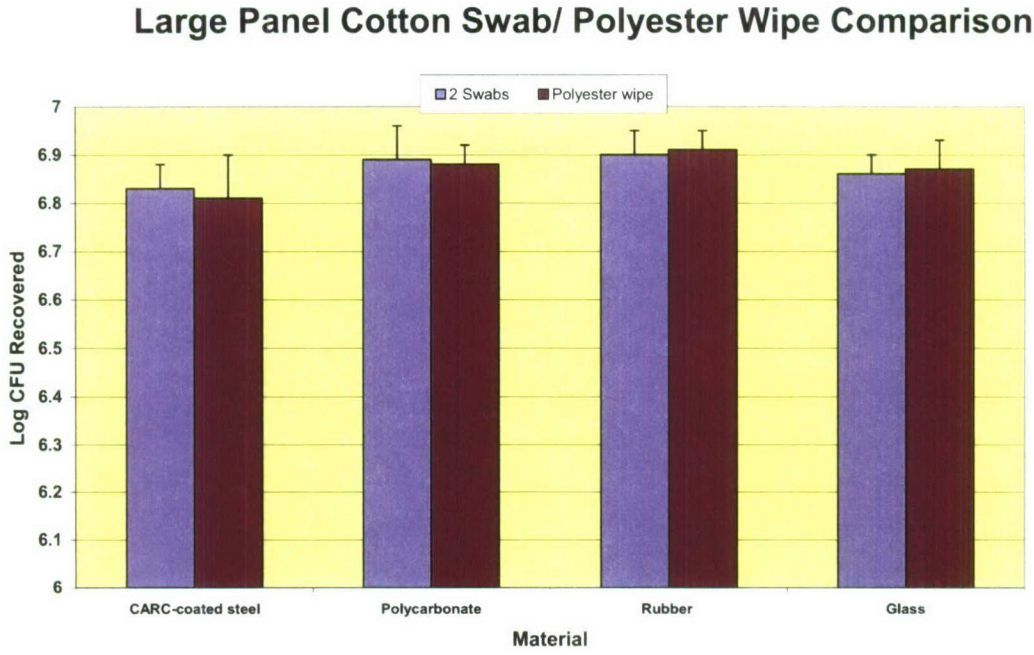

Figure 9. Spore Recovery Comparison with Wipes and Swabs from Large Panel.
Note expanded Y-scale for better depicting the subtle differences among log CFU.

3.5.2 Improved Sampling Protocol for Large Panels

The sampling-based spore recovery experiments from small coupons and large panels showed that residual spores remained on the surfaces after sampling with either one polyester wipe or swab. In order to retrieve the residual spores, experiments were designed to evaluate sampling with a combination of a single wipe followed by three successive cotton swabs. The spores recovered with one wipe and each of the three swabs were enumerated to assess log CFU and percentage recovery. The results summarized in Figures 12 and 13 show the relative spore recovery profile from all four panel types. Based on these results, use of one wipe and three successive swabs was determined to be the optimal spore recovery protocol from large panels.

3.6 Four Experimental Repeats for Spore Recovery from Large Panels

In order to assess spore recovery using the optimized sampling protocol, four sampling experimental repeats on large (12 x 12 in.) panels were performed by four laboratory analysts. These experiments were performed with each inoculant spore type, *B. subtilis* and ΔSterne. Each analyst sampled 16 pre-marked areas from one of four panel types per experiment; thus, the experimental design encompassed a randomized approach where, for each material, 64 replicate data points were generated. The results are summarized in Figure 14. The average recoveries from CARC-painted steel, polycarbonate, glass, and rubber, with both spore types, ranged between 65-75%, indicating that the optimized sampling method developed here yielded high spore recovery from both porous and non-porous panel surfaces.

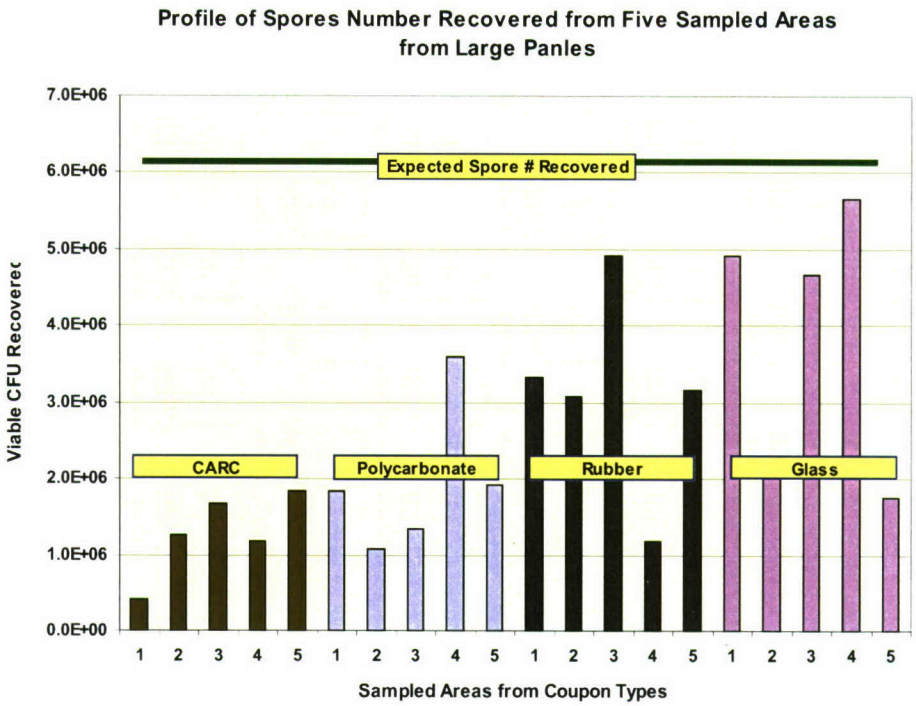

Figure 10. Spore Recovery Profile from Sampled Areas after Inoculation with 10 mL Spore Suspension

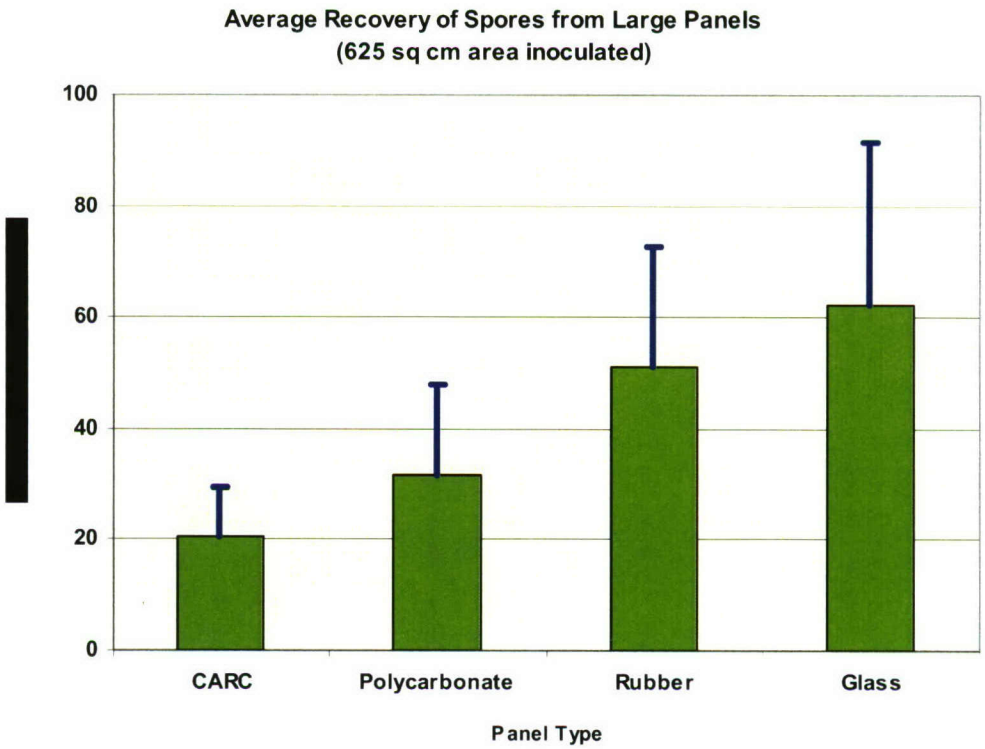

Figure 11. Spore Recovery Summary from Four Panel Types after 10-mL Inoculation

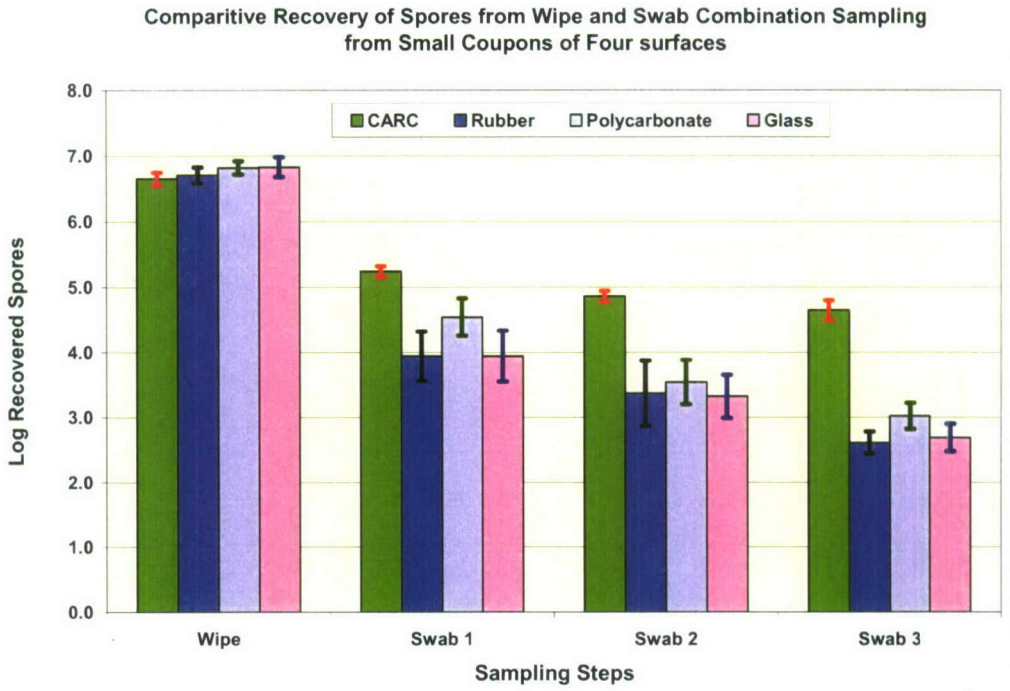

Figure 12. Spore Recovery with One Wipe and Three Swabs (data represented as log CFU recovered)

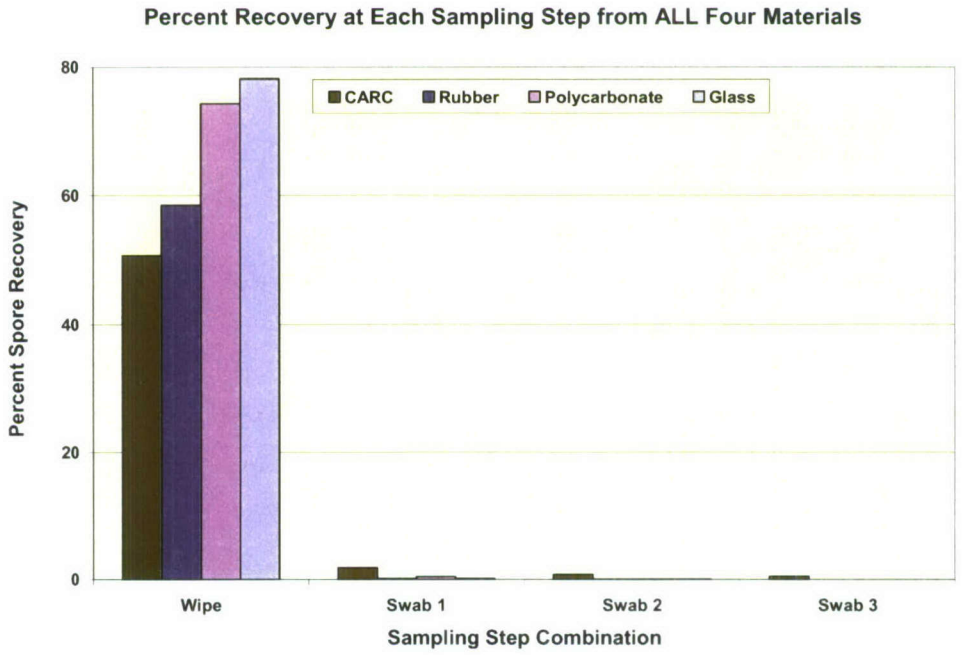

Figure 13. Spore Recovery with One Wipe and Three Swabs (data represented as a percentage of total spore inoculated).

3.7 Efficacy of CD Gas as Assessed by Sampling-Based Decon Protocol

The sporicidal efficacy of chlorine dioxide gas was assessed for both, *B. subtilis* and *B. anthracis* ΔSterne, on all four panel types. Six experimental repeats were performed for each spore type, with 16 sectors on each panel sampled after fumigation (test samples) and 4 sectors sampled before fumigation (positive samples). The fumigation was performed with ~9,000 ppmv.hr dose (CT = conc. X time in hours) at 75% RH. After six repeats, a total of 96 data points for test samples and 24 control samples was generated. The results were analyzed for both spore recovery and kill efficacy as indicated by log reduction of viable spores. Results with *B. subtilis* spores summarized in Figures 15 and 16 show 65-80% spore recoveries from all four panel types. A wide variability was observed in the efficacy of CD gas. The log reduction values were significantly lower for CARC-painted steel and butyl rubber (~4 log reduction with ~2 logs S.D.). Slightly higher log reduction values were seen for polycarbonate and glass (~5.5 log reduction with ~1 log S.D.).

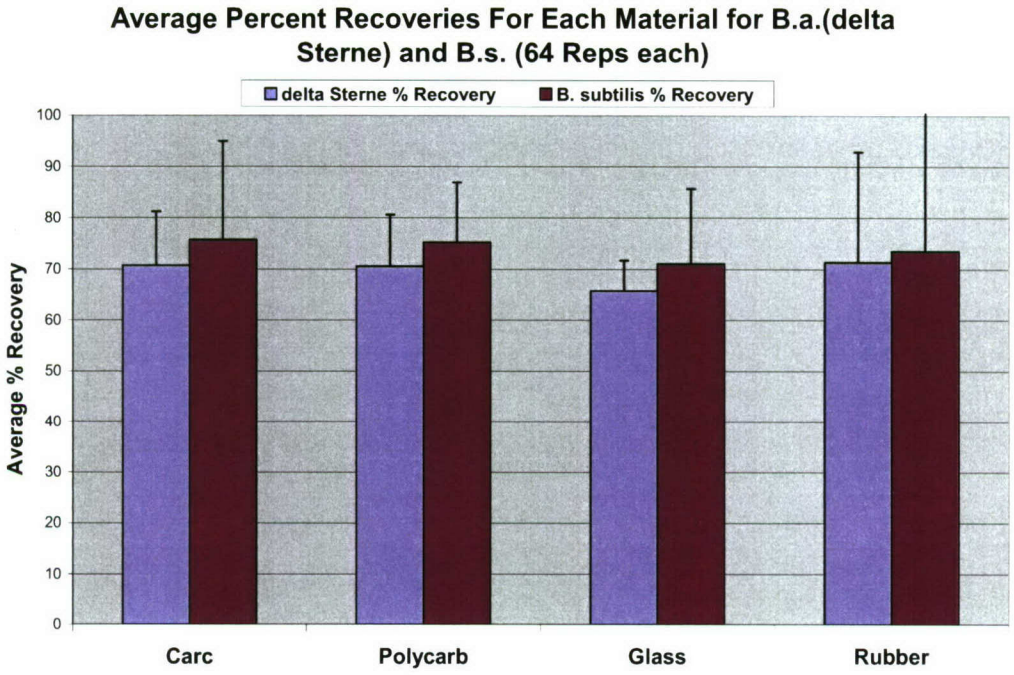

Figure 14. Summary of Spore Recovery from Four Panel Types (average of 64 data points).

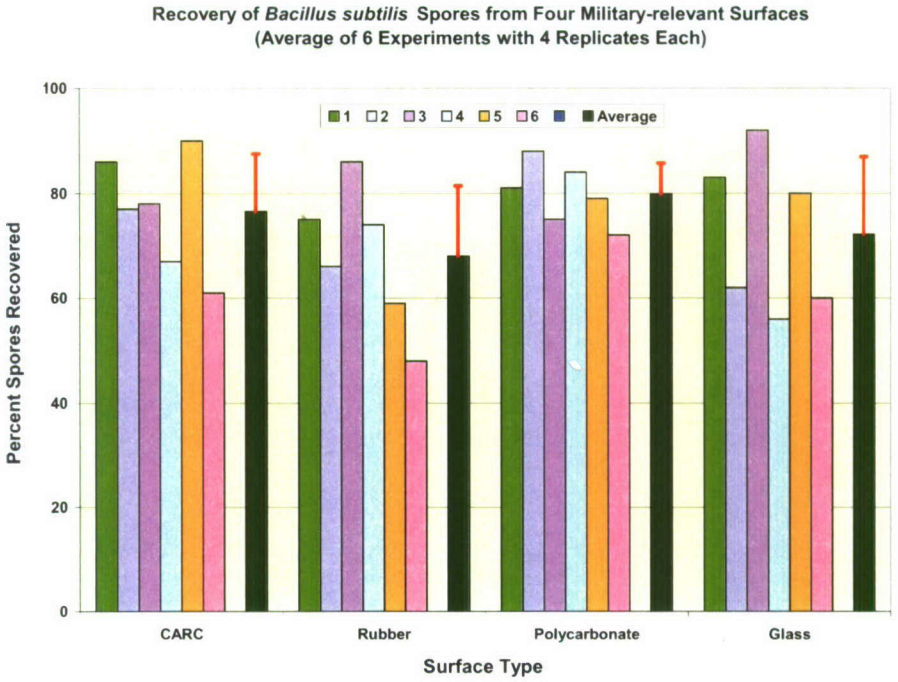

Figure 15. Summary of *B. subtilis* Spore Recovery in Six CD Fumigation Runs

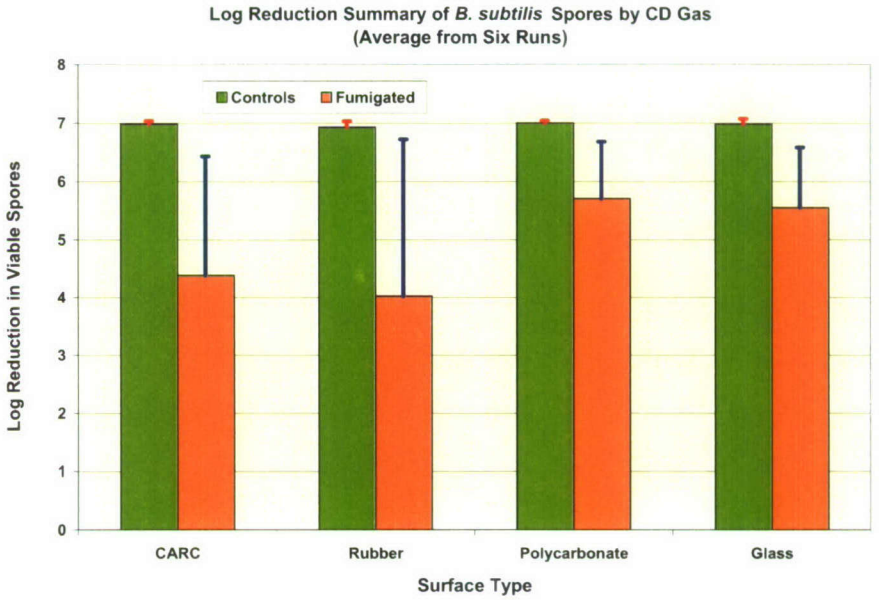

Figure 16. Sporicidal Efficacy of CD Gas against *B. subtilis* Spores

The sporicidal efficacy results of CD gas against Δ Sterne spores are summarized in Figures 17 and 18. Spore recoveries ranged between 62-83% from all panel types, with significant variability observed for rubber and polycarbonate. Similar to the *B. subtilis* results, the kill efficacy data for Δ Sterne showed high variability, with the highest kill observed for glass (5.9 log reduction ± 0.7 log S.D.). The lowest kill efficacy was observed for rubber panel, with a log reduction of 3.6 ± 1.5 log S.D.

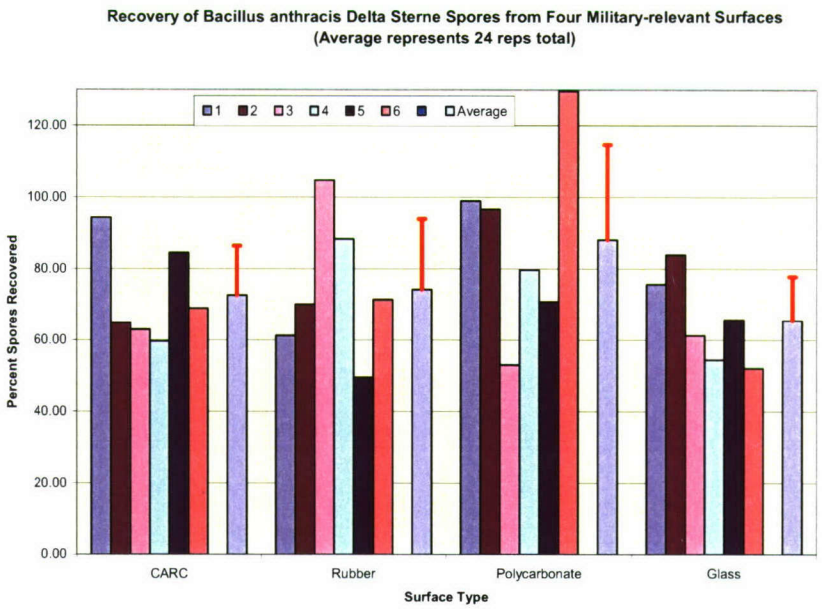

Figure 17. Summary of *B. anthracis* ΔSterne Spore Recovery in Six CD Fumigation Runs

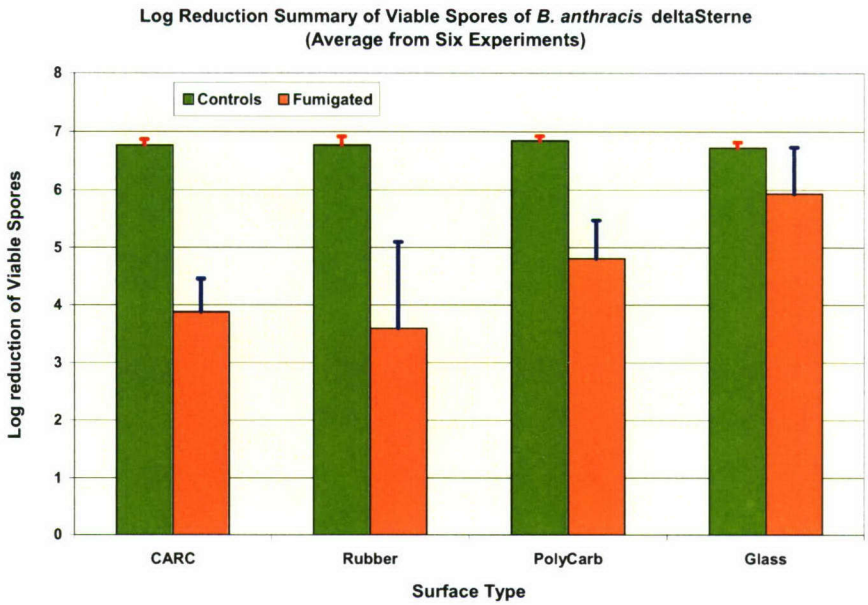

Figure 18. Sporicidal Efficacy of CD Gas against *B. anthracis* ΔSterne Spores

3.8 Sporicidal Efficacy of VHP as Assessed by Sampling-Based Decon Protocol

Decontamination experiments with VHP were conducted with all four panel types inoculated with either *B. subtilis* spores or ΔSterne spores. A dose of 1000-1200 ppmv.hr of VHP was used in these experiments. The spore recovery and kill efficacy results with *B. subtilis* are summarized in

Figures 19 and 20. The spore recovery for all six experiments was variable and ranged between 65-85%, with ±20% S.D. Lowest spore recovery was observed from rubber panels, whereas the highest recovery was seen from glass panels. In contrast to CD gas, ≥6 log reduction was observed for all panel types with low standard deviations.

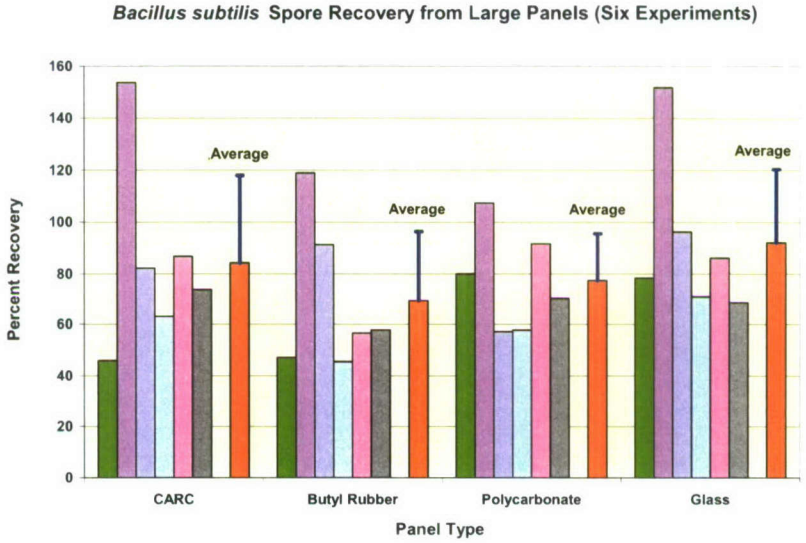

Figure 19. Summary of *B. subtilis* Spore Recovery in VHP Fumigation Runs

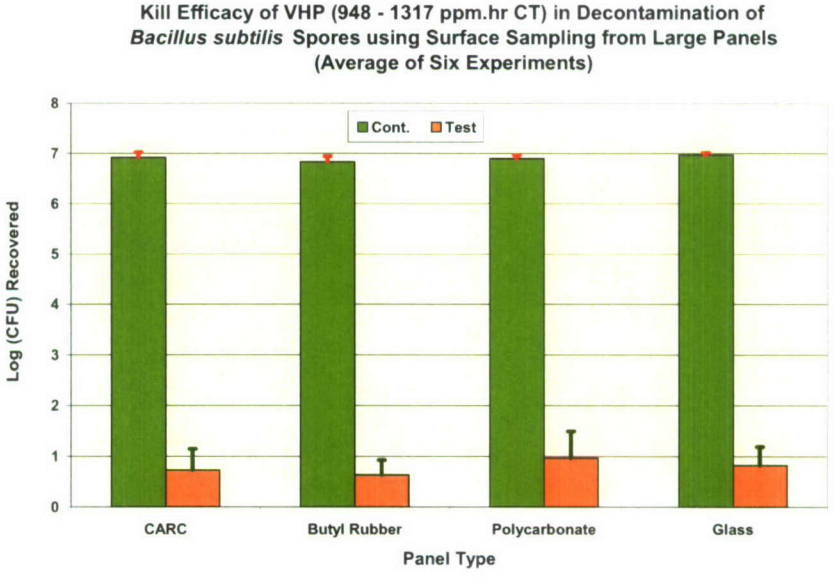

Figure 20. Sporicidal Efficacy of VHP against *B. subtilis* Spores

The results of VHP® testing against *B. anthracis* Δ Sterne are summarized in Figures 21 and 22. The spore recovery for all six experiments was variable and ranged between 63-83%, with ±20% S.D. Similar to the *B. subtilis* results, the lowest spore recovery was observed from rubber panels, whereas the highest recovery was seen from glass panels. As with *B. subtilis*, the kill efficacy showed low variability with 5.9-6.2 log reduction for Δ Sterne spores.

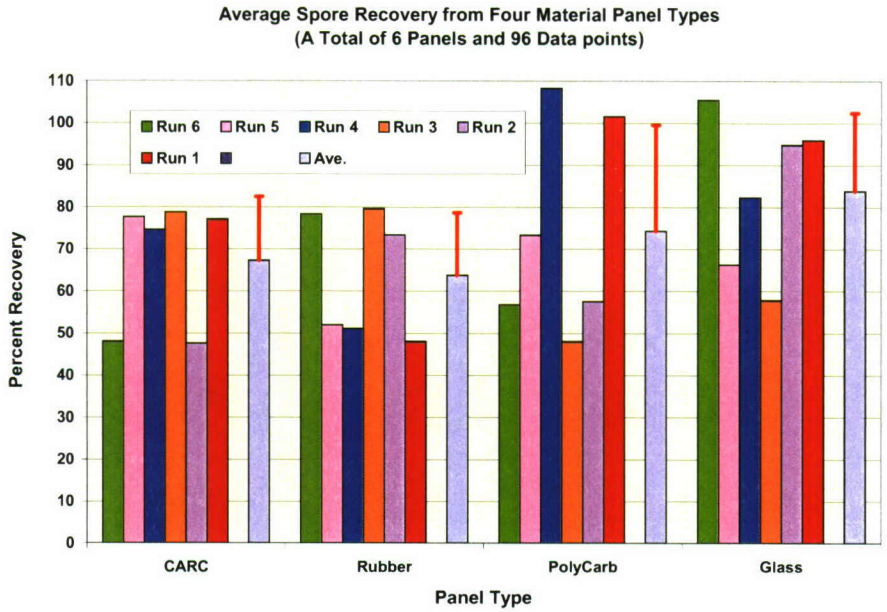

Figure 21. Summary of *B. anthracis* Δ Sterne Spore Recovery in Six VHP Fumigation Runs

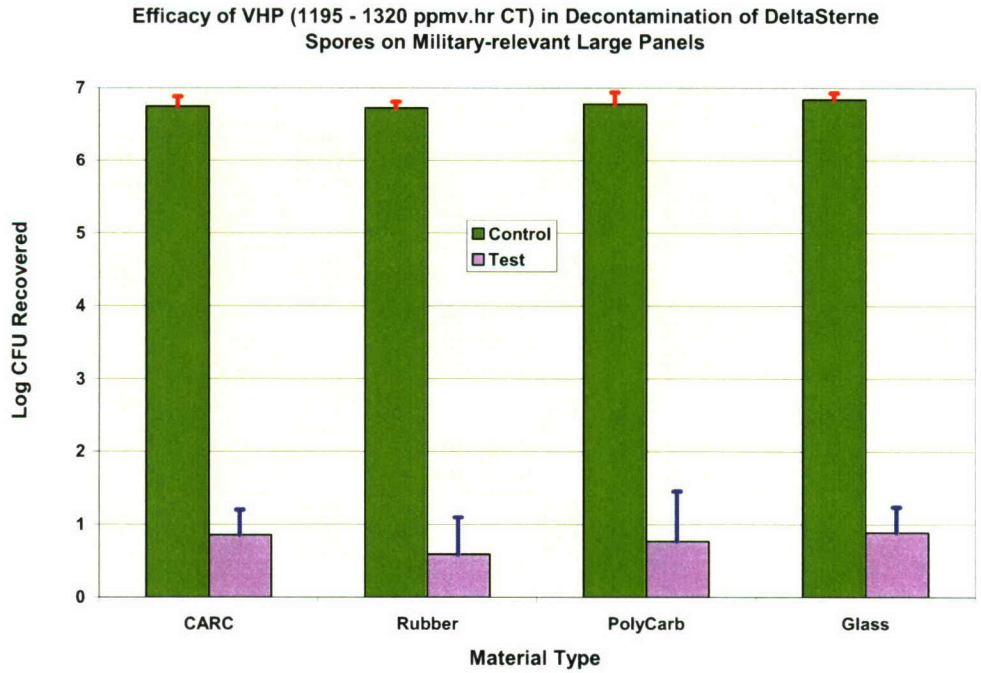

Figure 22. Sporicidal Efficacy of VHP Gas against *B. anthracis* ΔSterne Spores

4. DISCUSSION

A number of standardized test methods (ASTM 2414-05 and 2197-02) for determining the efficacy of liquid disinfectants and gaseous fumigants rely on the use of small-size carriers inoculated with test microbes. Typically, a small aliquot of spore suspension is dried on either hard porous or non-porous coupons, and these inoculated carriers are then exposed to disinfectants for a given time. Such methods are adequate for the screening and registration of test chemicals as sporicidal agents. However, utility of such decon products must be demonstrated on larger surface areas or 'real-life' test articles. The standardized test methods do not allow the user to assess efficacy of decon products on surfaces larger than 1-2 cm in size. Pre- and post-decon assessment of viable spores from large surfaces require adequate sampling methodologies, with high spore recoveries from porous and non-porous surfaces relevant to military operations. The principal objective of this program was to develop a sampling-based decon protocol for assessing the efficacy of liquid disinfectants and gaseous fumigants for four select military-relevant surfaces, glass (as a control), butyl rubber (representative of masks), polycarbonate (representative of mask lenses), and CARC-painted steel (representative of military equipment). The testing with liquid disinfectants was performed by our collaborators in Dahlgren and the data are not included in this report. Here, we summarize the results obtained in the context of sporicidal efficacy of gaseous fumigants.

As a prelude to the development of such a decon protocol, a number of parameters were optimized:

- Spore extraction media from small size coupons.
- Effect of two common physical treatments, vortexing and sonication for dislodging spores off the small coupons.
- Effect of different inoculation methods on spore recovery.
- Screening of two wipe types and two swab types as surface sampling materials.
- Surface sampling optimization from large panels with respect to maximal spore recovery.

Based on the published literature, sampling techniques result in low (10-50%) recoveries of biological materials (Sanderson *et al.*, 2002; Rose *et al.*, 2004; and Brown *et al.*, 2007). The data presented in this report clearly provide evidence for spore recoveries in the range of 60-90%, which is significantly higher for porous surfaces. Interestingly, while extraction of ΔSterne spores required use of BPW with surfactant, spores of *B. subtilis* required just use of water. This is a significant difference between the extraction processes of two spore types. This difference could be due to differences in surface charge and/or hydrophobicity of the two spore types.

Availability of sampling protocols with high spore recovery is absolutely vital to development of an improved decontamination protocol for large surfaces. In a comparison of two commonly used physical extraction approaches for releasing spores off coupon surfaces or from wipes or swabs, vortexing and sonication, vortexing alone was found to be adequate to efficiently release spores from each surface/material type. In addition, spore extraction in a single tube optimized here allows high-throughput scale up and process stream-lining.

Efficacy of CD gas and VHP were evaluated at a fixed dose of ~9000 ppmv.hr and 1200 ppmv.hr. It appears that for the four material types, VHP is more efficacious in the decontamination of both spore types (>6 log kill). In contrast, CD gas resulted in 3-5 log spore kill, depending on the material type. Effective decontamination of such surfaces may require a significantly higher dose of CD gas (>9,000 ppmv.hr). Further work is required to optimize effective decontamination of the four material

types by CD gas, e.g., longer exposure times and/or slightly higher RH. Broader applications of the protocol developed here require further work using additional porous and non-porous surfaces relevant to military applications.

5. CONCLUSIONS

The purpose of this program was three-fold: a) review and identify current-bio-decon protocols for evaluating efficacy of liquid and gaseous sporicidal agents; b) optimize various parameters for spore extraction from small size coupons (2x5 cm) and for surface sampling from small coupons and large panels (12x12 in.) prepared from four military-relevant surfaces, i.e., glass, polycarbonate, CARC-painted steel, and butyl rubber; and c) develop a high-throughput sampling-based decon protocol for determining the efficacy of decon agents. Our collaborators (Lindsay Sobota and her group at NSWC) initially focused on *B. subtilis* and our group at ECBC focused on ΔSterne. After initial optimization of conditions with respect to spore inoculation and extraction by Dahlgren and Edgewood, experiments were repeated in each of these laboratories with the other spore strain, i.e., Dahlgren with ΔSterne and Edgewood with *B. subtilis*. The two laboratories, therefore, confirmed initial optimization results on spore extraction and spore sampling from small coupons and large panels. Interestingly, while water was an adequate extractant for *B. subtilis* spores, extraction of ΔSterne was optimal in BPW containing surfactant. With respect to sampling, polyester wipes and cotton swabs were found to retrieve maximal spores off porous and non-porous surfaces. Vortexing was found to be adequate for spore extraction from small coupons and the release of spores from sampling materials, i.e., wipes and polyester. The spore recovery from CARC-painted steel and butyl rubber was consistently less than from glass and polycarbonate. Overall, the spore recovery by either extraction or sampling was higher than most published reports, and it ranged between 60-100%.

In the latter part of the program, the Dahlgren lab focused on efficacy studies with liquid disinfectants and the Edgewood lab focused on gaseous fumigants. Significant amounts of spores were found to be left on the surface of CARC-painted steel and butyl rubber. In order to further improve the spore recovery from all large panels, use of one polyester wipe and three swabs was found to retrieve >60% spores off even a highly porous surface, CARC-painted steel. Out of the 20 sectors inoculated, four were sampled before fumigation and 16 were sampled post-fumigation. The efficacy of VHP (with 1200-100 ppm.hr) and CD gas (9000 ppmv.hr) was determined with *B. subtilis* and ΔSterne. For the four military-relevant surfaces, VHP was found to be highly efficacious in six-log spore kill. In contrast, CD gas was less effective with both inoculant types, especially on CARC-painted rubber and butyl rubber, as 3.5-4 log spore kill was observed for the two inoculants. The spore kill on polycarbonate was slightly better (4.8-5.7 log kill), but the spore kill on glass gave the best result, i.e., between 5.5 and 6. Overall, VHP resulted in >6 log kill and CD resulted in <6 log kill.

Further work is recommended on the sampling-based decon protocol developed to asses the efficacy of VHP and CD gas on large surfaces. Future testing should include additional porous surfaces relevant to DoD and spores from other virulent strains of *B. anthracis* with varied doses of VHP and CD gas.

LITERATURE CITED

1. ASTM International. *Standardized Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporicidal Activities of Liquid Chemical Germicides*; Standard Test Method E 2197-02; American Society for Testing and Materials: West Conshohocken, PA, 2002.

2. ASTM International. *Standard Test Method E 2414-05; Quantitative Sporicidal Three-Step Method (TSM) to Determine Sporicidal Efficacy of Liquids, Liquids Sprays, and Vapor or Gases on Contaminated Carrier Surfaces*; American Society for Testing and Materials: West Conshohocken, PA, 2005.

3. Brown, G.S.; Betty, R.G.; Brockman, J.E.; Lucero, D.A.; Souza, C.A.; Walsh, K.S.; Boucher, R.M.; Tezak, M.; Wilson, M.C.; Rudolph, T. Evaluation of a Wipe Surface Sample Method for Collection of *Bacillus* Spores from Nonporous Surfaces. *Appl. Environ. Microbiol.* **2007**, *73*, 706-710.

4. Canter, D.A. Addressing Residual Risk Issues at Anthrax Cleanup. How Clean Is Safe. *J. Toxicol. Environ. Health Anal.* **2005**, *68*, 1017-1032.

5. Centers for Disease Control and Prevention. Procedures for Collecting Surface Environmental Samples for Culturing *B. Anthracis*. http://www.Bt.Cdc.Gov/Agent/Anthrax/Environmental-Sampling-Apr2002.Asp. (accessed February 2006).

6. US Government Accountability Office. *Anthrax Detection – Agencies Need to Validate Sampling Activities in order to Increase Confidence in Negative Results*; GAO-05-493T; Government Accountability Office: Washington, DC, 2005.

7. Hodges, L.R.; Rose, L.J.; Peterson, A.; Noble-Wang, J.; Arduino, V. Evaluation of Macrofoam Swab Protocol for the Recovery of *Bacillus anthracis* Spores from a Steel Surface. *Appl. Environ. Microbiol.* **2006**, *72*, 4429-4430.

8. Inglesby, T.V.; Henderson, D.A.; Bartlett, J.G.; Ascher, M.S.; Eitzen, E.; Friedlander, A.M.; Hauer, J.; Mcdade, J.; Osterholm, M.T.; O'Toole, T.; Parker, G.; Perl, T.M.; Russell, P.K.; Tonat, K. Anthrax as a Biological Weapon, Medical and Public Health Management. *JAMA* **1999**, *281*, 1735-1745.

9. AOAC International Method 966.04; *Official Methods of Analysis;* 21st ed.; Chapter 6; AOAC International: Gaithersburg, MD, 2006.

10. Rose, L.; Jensen, B.; Peterson, A.; Banerjee, S.N.; Arduino, M. Swab Materials and *Bacillus anthracis* Spore Recovery from Nonporous Surfaces. *Emerg. Infect. Dis.* **2004**, *10*, 1023-1029.

11. Sanderson, W.T.; Hein, M.J.; Taylor, L.; Curwin, B.D.; Kinnes, G.M.; Seitz, T.A.; Popovic, T; Holmes. H.T.; Kellum, M.E.; McAllister, S.K.; Whaley, D.N.; Tupin, E.A.; Walker, T.; Freed, J.A.; Small, D.S.; Klusaritz, B.; Bridges, J.H. Surface Sampling Methods for *Bacillus anthracis* Spore Contamination. *Emerg. Inf. Dis.* **2002**, *8*, 1145-1151.

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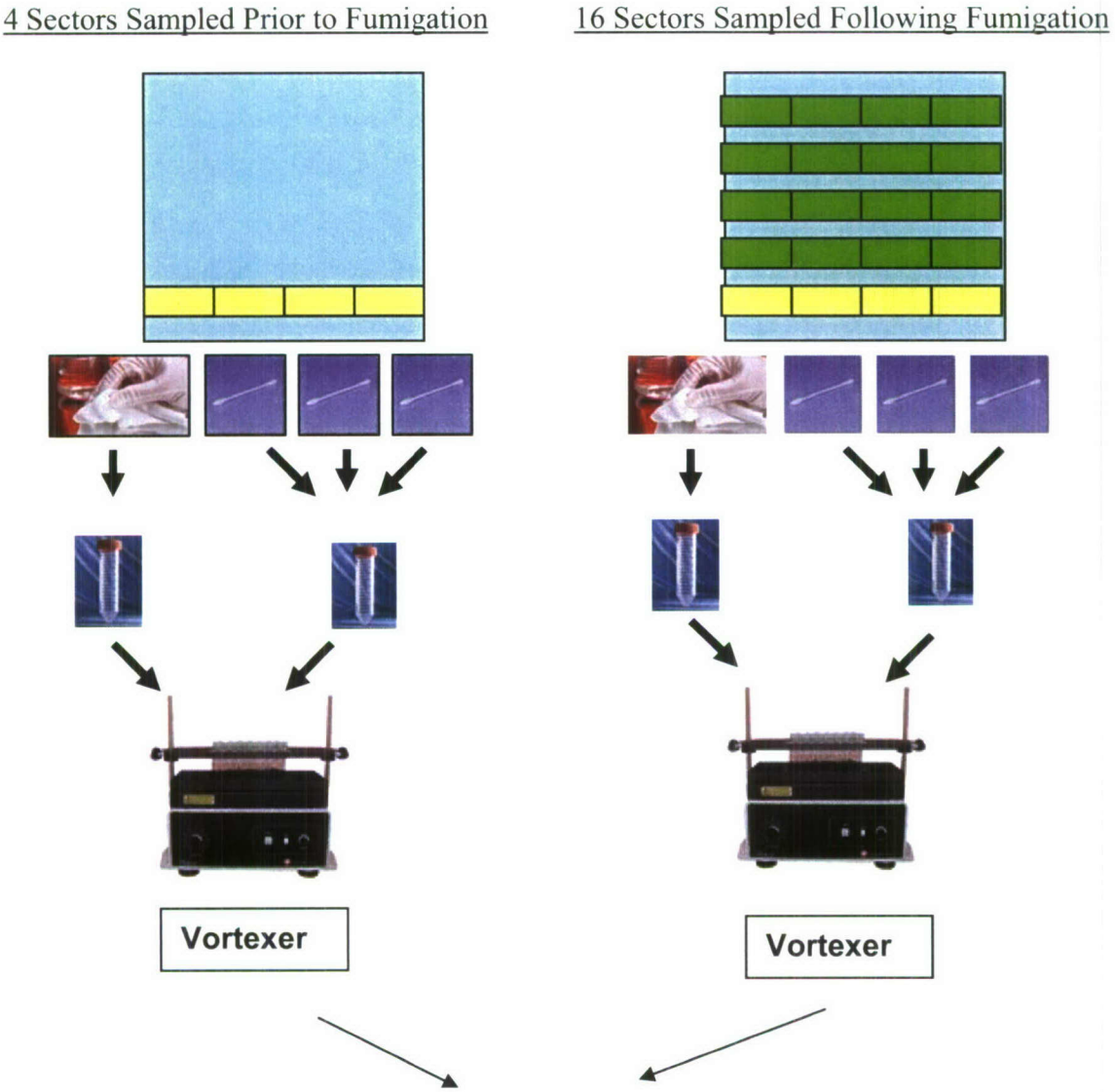
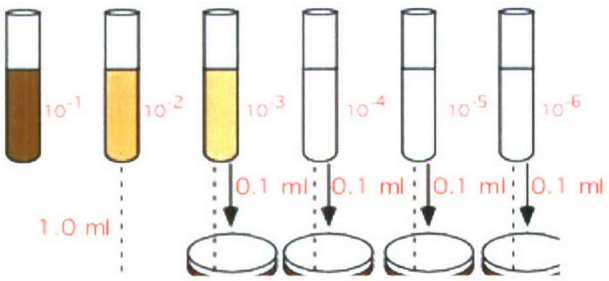
APPENDIX A
FLOW CHART OF SAMPLING-BASED DECONTAMINATION PROTOCOL



Dilution Plating



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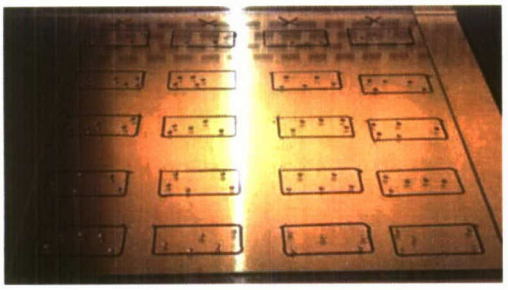
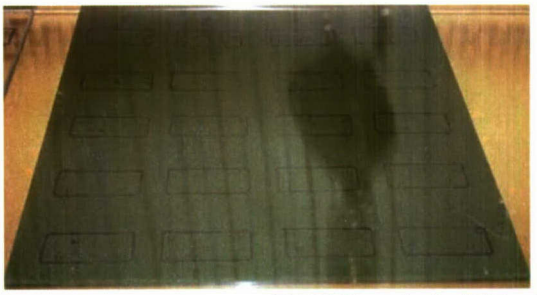
APPENDIX B
INOCULATED PANELS, SAMPLING WITH WIPES AND SWABS,
PANELS IN THE FUMIGATION CHAMBER, AND THE FUMIGATION CHAMBER

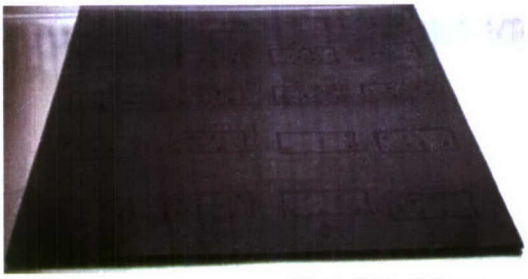
Inoculated Panels



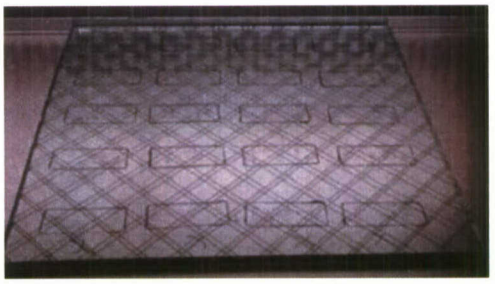
Polycarbonate



CARC-painted Steel

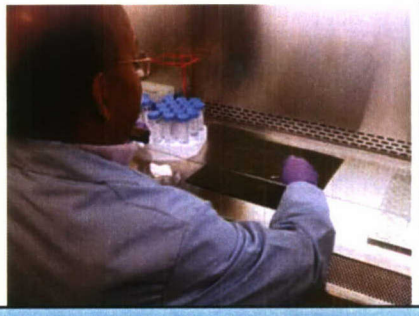


Butyl Rubber



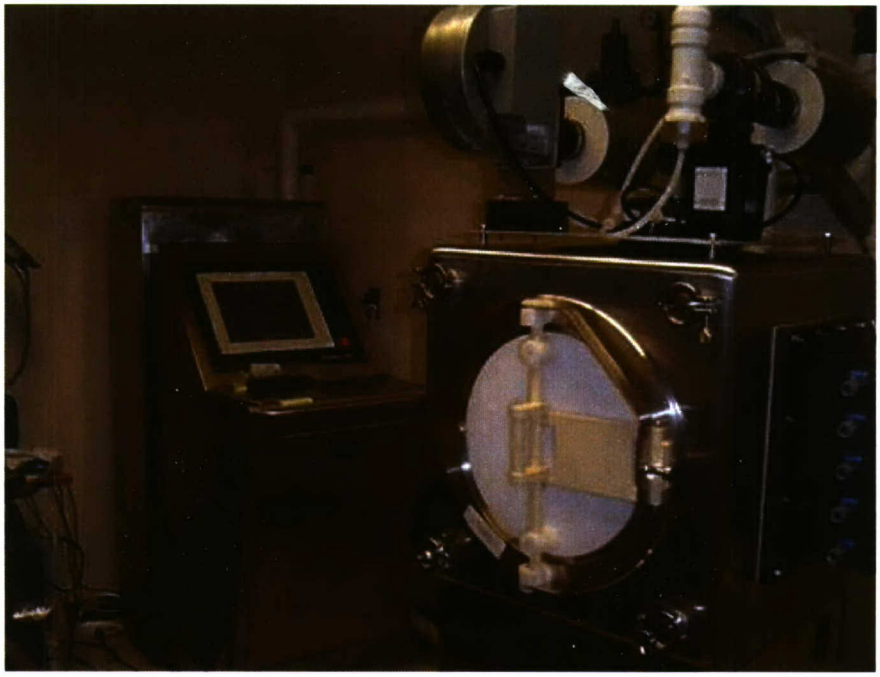
Glass

Sampling from large Panels



Sampling with WIPE (1) Swabs (3) Panels in the Chamber

Fumigation Chamber and ClorDiSys CD Generator (8 cubic ft in size)



APPENDIX C
PLATE COUNTING

QCount™